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Troglitazone halts diabetic glomerulosclerosis by blockade of mesangial expansion[1]

Kevin John McCarthy, Robert Earl Routh, Walter Shaw, Kathleen Walsh, Tomas Charles Welbourne, and John Hardwick Johnson

School of Medicine, Louisiana State University Medical Center, Shreveport, Louisiana; Genetic Models Inc., Indianapolis, Indiana; Parke Davis Division, Warner Lambert Inc., Ann Arbor, Michigan, USA

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Background. Renal complications of long-term, poorly controlled type 2 diabetes mellitus include glomerulosclerosis and interstitial fibrosis. The onset and progression of these complications are influenced by underlying pathophysiologies such as hyperglycemia, hypertriglyceridemia, and hypercholesterolemia. Troglitazone, a thiazolidinedione, has been shown to ameliorate these metabolic defects. However, it was not known whether therapeutic intervention with troglitazone would prevent the onset and progression of glomerulosclerosis.

Methods. Sixty male ZDF/Gmi™ rats and 30 age-matched Zucker lean rats were in the study. The ZDF/Gmi™ rats were divided into two groups, one in which blood glucose levels were uncontrolled (30 animals) and another (30) in which blood glucose was controlled via dietary administration of troglitazone. Ten animals from each group were sacrificed at one, three, and six months into the study. The kidneys were harvested and processed for immunostaining with BM-CSPG, a marker for mesangial matrix. Images of 200 glomeruli per animal were captured using digital imaging microscopy, and the index of mesangial expansion (total area mesangium/total area of tuft) per glomerular section was measured.

Results. The administration of troglitazone ameliorated the metabolic defects associated with type 2 diabetes mellitus. Moreover, the glomeruli from tissue sections of animals given troglitazone showed no mesangial expansion when compared with normoglycemic control animals, whereas the uncontrolled diabetic animals showed significant mesangial expansion at all time intervals.

Conclusions. Therapeutic intervention with the thiazolidinedione troglitazone halts the early onset and progression of mesangial expansion in the ZDF/Gmi™ rat, preventing the development of glomerulosclerosis in this animal model of type 2 diabetes mellitus.

[1] **See Editorial by Steffes, p. 2592**

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The progression of renal complications associated with long-term, poorly controlled diabetes mellitus occurs in humans over a time course of 10 to 25 years. The histoarchitectural changes associated with disease progression are well described in the literature and include glomerular hypertrophy, thickening of the glomerular basement membrane (GBM), mesangial expansion, afferent and efferent arteriolar hyalinosis, and interstitial fibrosis [1–3]. These structural changes have been shown to correlate with alterations in synthesis and degradation of the macromolecular constituents of the extracellular matrices comprising the glomerulus and associated tissues [4–20].

Thickening of the GBM is known to occur in individuals in the early stages of diabetes mellitus [21–23] and has been thought to contribute to the proteinuria seen in diabetes. However, the description of a potentially more critical lesion, that is, the progressive disruption of the ordered extracellular matrix domains within the glomerulus known as mesangial expansion, was first provided in the elegant morphologic studies of both Osterby [23, 24] and Steffes and Mauer [25–27]. Earlier immunohistochemical studies from our laboratory corroborated those results in part by showing that a macromolecular constituent of the normal mesangial matrix basement membrane-specific chondroitin sulfate proteoglycan [28] (BM-CSPG; referred to as bamacan [29]) becomes abnormally associated with the glomerular capillary wall [30] in an animal model of type 1 diabetes mellitus, possibly as a result of uncontrolled mesangial expansion.

In this study, we return to the issue of mesangial expansion in the male Zucker diabetic fatty (Gmi ZDF fa/fa) rat, a model of type 2 diabetes. The male ZDF rat is an obese, insulin-resistant, and hyperinsulinemic animal that develops impaired glucose tolerance by six weeks of age. By 12 weeks of age, β-cells fail to secrete insulin in response to glucose, resulting in hyperglycemia and type 2 diabetes [31]. Early intervention with an agent that increases insulin sensitivity, such as troglitazone, prevents the develop-

ment of hyperglycemia, normalizes glucose tolerance, and corrects the dyslipidemia in this model [32].

Troglitazone has been reported to correct albuminuria in streptozotocin-treated diabetic rats [33]. We therefore examined the long-term effects of troglitazone treatment on the progression of renal histopathology in the spontaneously diabetic ZDF rat, using the pattern of immunohistochemical staining of BM-CSPG as an indicator of mesangial area/matrix domain within a glomerulus in order to determine whether therapeutic intervention with troglitazone would prevent the entry/progression of glomeruli into the sclerotic process.

METHODS

Animals and animal care

Zucker diabetic fatty rats (ZDF/Gmi™ fa/fa; Genetic Models Inc., Indianapolis, IN, USA) were fed a diet of Purina 5008 chow with or without troglitazone (ZDF/TRO) as an admixture: 6 mg/g chow for the first 12 weeks and 12 mg/g chow between weeks 13 and 25. Because of the gain in weight of the troglitazone-treated animals during the first 12 weeks, the adjustment in the amount of troglitazone in the diet during weeks 13 through 25 was done to maintain the dose of troglitazone in the therapeutic range of the drug. Troglitazone treatment was begun when the animals were six weeks of age. Controls were lean Zucker littermates (ZL = ZDF/Gmi fa/+ or +/+; Genetic Models Inc.) fed Purina 5008 without troglitazone. Animals were housed in animal quarters maintained at 22°C with a 12- to 12-hour fixed light-dark cycle. Blood glucose, triglycerides, and insulin were monitored weekly. Blood was collected from the tail vein, and cholesterol and glucose were measured on a Beckman analyzer. Triglycerides were measured colorimetrically using a Boehringer Triglycerides assay (Boehringer Mannheim, Indianapolis, IN, USA). Insulin concentrations were determined on duplicate samples using radioimmunoassay (Linco Research, Inc., St. Charles, MO, USA).

Morphology

Immunological reagents. The characterization of core protein-specific monoclonal antibodies for BM-CSPG, a chondroitin sulfate proteoglycan present in the mesangial matrix, has been previously described [28, 34]. Secondary antibodies (goat anti-mouse IgG) conjugated to fluorescein were purchased from Jackson Immunoresearch (Malverne, PA, USA). The discrete immunolocalization of BM-CSPG to the mesangial matrix allowed us to use this tool to (*1*) identify those glomeruli that had entered the sclerotic pathway, and (*2*) track and measure the degree of mesangial expansion in glomeruli in kidney sections from each animal.

Light microscopy specimen preparation. The kidneys were harvested at necropsy. To enhance tissue penetration of fixative, two transverse cuts made in the kidney flanking the pelvis, and the entire kidney was fixed by immersion fixation in Clark's solution (75% ethanol and 25% acetic acid) [35]. Postfixation, the kidneys were removed from the fixative, blotted to remove excess liquid, weighed, and subsequently processed for paraffin embedding as previously described [28, 34]. Specimen preparation for digital imaging microscopy studies was as follows. In order to maximize the number of potential targets/slide, during microtomy, each tissue section was collected in steps of 500 μm, that is, section 2 on the slide was 500 μm deeper into the block than section 1. Four 4 μm tissue sections were mounted per slide; therefore, each microscope slide represented a 2 mm excursion into the tissue specimen. Single-label immunohistochemistry for BM-CSPG was performed as previously described [34, 36]. The same lot of monoclonal antibody and secondary antibody was used for all observations during the course of the study to minimize error in staining intensities caused by lot-to-lot variations in antibody titer.

Digital image acquisition

Immunostained slides were observed using a Leitz Ortholux microscope equipped for epifluorescent illumination. All glomeruli were imaged using a ×40 objective magnification. A total of 200 glomeruli was digitized for each animal. The glomeruli were targeted and acquired in a sequential manner starting from the outer cortex, moving in a linear path toward the corticomedullary junction, then moving one field laterally, and then moving back toward the outer cortex. This scanning pattern was repeated until a total of 200 glomeruli per animal was acquired. Glomeruli that appeared to be cut tangentially were excluded from the observation group. Images of glomeruli were digitized using a Dage-MTI (Michigan City, IN, USA) CCD-100 monochromatic camera, the signal from which was ported to a PowerPC 9600 (Apple Computer Corp., Cupertino, CA, USA) hosting an LG-3 imaging board (Scion Corp., Frederick, MD, USA). Both image acquisition and data analysis were performed using the imaging software, IPLabSpectrum (Scanalytics Corp., Fairfax, VA, USA).

Image analysis and morphometry measurement of glomerular area (At) were performed by manually tracing the perimeter of the capillary tuft cut in transverse section using a linetool subroutine resident within the imaging software package. The area of BM-CSPG immunoreactivity within the arborized region of the mesangium was used to delineate the extent of mesangium within each glomerular section (Am). The measurement of the area of BM-CSPG immunostaining was performed using a segmentation tool resident within the imaging software package. The mean glomerular volume was estimated using this equation:

Table 1. Physiological parameters

	Body weight	Kidney weight	Body wt/kidney wt	Blood glucose (nonfasting)	Insulin	Triglycerides	Cholesterol
	g			*mg/dL*	*ng/dL*	*mg/dL*	
Normal range				(90–200)	(0.5–4.0)	(50–150)	(50–120)
ZL (t = 0)	146 ± 3	ND	ND	137 ± 3	0.78 ± 0.13	74 ± 1	81 ± 6
Obese ZDF (t = 0)	155 ± 6	ND	ND	146 ± 4	5.87 ± 0.62[a]	80 ± 2[b]	152 ± 12[a]
ZL (t = 4 weeks)	288 ± 4	ND	ND	131 ± 1	1.08 ± 0.17	139 ± 8	62 ± 1
Obese ZDF (t = 4 weeks)	341 ± 4[a,c]	ND	ND	447 ± 25[a,d]	13.32 ± 1.26[a]	1107 ± 66[a,d]	90 ± 3[a]
Obese ZDF troglitazone-treated (t = 4 weeks)	396 ± 9[a]	ND	ND	131 ± 2	8.86 ± 0.62[a]	120 ± 5	86 ± 3[a]
ZL (t = 12 weeks)	389 ± 8	1.52 ± 0.03	0.0039	143 ± 3	1.4 ± 0.3	134 ± 8	58 ± 1
Obese ZDF (t = 12 weeks)	444 ± 7[a,c]	2.19 ± 0.06	0.0049[a]	675 ± 25[a,d]	3.5 ± 0.4[d]	813 ± 85[a,d]	157 ± 4[a,c]
Obese ZDF troglitazone-treated (t = 12 weeks)	657 ± 14[a]	1.94 ± 0.07	0.0028	162 ± 3[b]	20.9 ± 1.6[a]	362 ± 16[b]	90 ± 4[b]
ZL (t = 25 weeks)	450 ± 7	1.51 ± 0.03	0.0034	143 ± 3	1.5 ± 0.4	228 ± 9	60 ± 1
Obese ZDF (t = 25 weeks)	445 ± 8[d]	2.87 ± 0.09	0.0064[a]	584 ± 18[a,d]	2.1 ± 0.2[d]	1704 ± 243[a,c]	242 ± 25[a]
Obese ZDF troglitazone-treated (t = 25 weeks)	810 ± 16[a]	2.05 ± 0.06	0.0025	140 ± 3	5.8 ± 0.5[a]	331 ± 26	106 ± 5[a]

Abbreviation: ND, not done.
[a] $P < 0.001$ compared to ZL
[b] $P < 0.05$ compared to ZL
[c] $P < 0.05$ compared to troglitazone-treated obese ZDF
[d] $P < 0.001$ compared to troglitazone-treated obese ZDF

$$V_G = \beta/K \times [A_G]^{3/2}$$

where $\beta = 1.38$ (sphere constant) and K (a distribution coefficient) = 1.10 [37, 38]. Mesangial volume fraction (V_vMes), an index of mesangial expansion, was automatically calculated by the image analysis software as the ratio Am/At [39].

Statistics

Differences between treatment groups were determined using the analysis of variance (ANOVA) subroutine in the application Statview (SAS, Cary, NC, USA), and statistical significance between groups was determined using Bonferroni/Dunn's test within the same application.

RESULTS

Animal body weight

Animal weight for all groups is shown in Table 1. On normal chow, diabetic ZDF animals were significantly heavier than lean controls at all ages ($P < 0.001$). ZDF/TRO animals were significantly heavier than either the untreated ZDF animals ($P < 0.05$) or ZL control animals ($P < 0.001$) after 4 weeks and remained so after 12 weeks ($P < 0.05$ and $P < 0.001$ for untreated ZDF and ZL animals, respectively) of treatment. Differences between ZDF/TRO animals and ZL controls or ZDF diabetic animals were magnified by 25 weeks. ZDF diabetic animals had begun to lose weight between weeks 21 and 25, and by week 25, there was no difference between the ZL and ZDF groups.

Troglitazone ameliorates hyperglycemia long term

Fasting hyperglycemia was observed in all untreated ZDF rats by three weeks (Table 1). Blood glucose levels in these animals were significantly different from both ZL animals ($P < 0.001$) and ZDF/TRO animals ($P < 0.001$). Blood glucose levels from ZL and ZDF/TRO animals were not significantly different. After 12 weeks of study, the average fasting blood glucose rose to 675 ± 25 mg/dL in the untreated ZDF group. The ZL lean animals had glucose levels of 143 ± 3 mg/dL ($P < 0.001$ vs. untreated ZDF). ZDF/TRO animal blood glucose values averaged 162 ± 3 mg/dL ($P < 0.001$ vs. untreated ZDF rats and P = NS vs. ZL). By week 25, ZDF diabetic animals remained hyperglycemic, while blood glucose from ZL and ZDF/TRO animals was virtually indistinguishable (Table 1).

Troglitazone ameliorates hyperinsulinemia long term

Insulin levels in the ZDF animals were significantly elevated relative to the ZL controls at the beginning of the study (Table 1). At four weeks, the insulin levels of the untreated ZDF animals had risen from 5.87 ± 0.62 to 13.32 ± 1.26 ng/dL. These values were significantly different from the ZL control ($P < 0.001$) and the ZDF/TRO ($P < 0.001$). Also, insulin values from the ZL animals were significantly different from the troglitazone-treated ZDF ($P < 0.001$). By week 12, insulin levels in ZDF diabetic animals had fallen to levels that were not different from the control ZL animals. This is because of well-documented β-cell failure in this model [31]. Circulating insulin levels in the ZDF/TRO animals continued to rise until 12 weeks (they were 20.9 ± 1.6 ng/dL). This value significantly differed from the values for the ZL

controls and the untreated ZDF animals ($P < 0.001$). During the first 12 weeks of the study, a gradual weight gain in the animals was observed, despite the fact that the troglitazone-treated animals maintained a consistent level of food intake. This unexpected weight gain, coupled with no apparent change in dietary intake, led to a net reduction in the troglitazone dose per gram of body weight. To correct for this situation, at week 12 the dose of troglitazone was increased to compensate for this weight gain, and the circulating insulin levels began to drop within a week (data not shown). Insulin levels remained stable at the troglitazone-treated animals at 5 to 6 ng/dL for the duration of the experiment. It should be emphasized that despite this lower effective dose and the rise in circulating insulin, these animals remained normoglycemic (Table 1). At the 25-week time point, circulating insulin levels in the ZDF/TRO animals were 5.8 ± 0.5 ng/dL. This value was significantly higher than the value for ZL and ZDF diabetic animals ($P < 0.001$). ZL and ZDF diabetic animals did not differ from one another at 25 weeks.

Troglitazone ameliorates hypertriglyceridemia long term

At the beginning of the study, triglyceride levels from obese animals were higher than in the ZL controls (Table 1). By week 4, untreated ZDF animals had circulating triglycerides almost tenfold higher than were found in either ZL or ZDF/TRO animals ($P < 0.001$). Triglycerides were slightly decreased at 12 weeks in the untreated ZDF animals (because of a very low triglyceride level in one animal), but increased significantly ($P < 0.001$) at 25 weeks. Circulating triglycerides were not different between ZL and ZDF/TRO animals at 4 weeks, but were significantly different at 12 weeks (134 ± 8 vs. 362 ± 16 mg/dL). This may have been due to the lower troglitazone dose. At 25 weeks, there was no difference between ZL ZDF/TRO animals (Table 1).

Also shown in Table 1 are the results of cholesterol determinations. Cholesterol levels in ZDF and ZDF/TRO animals were significantly higher than the ZL animals at all time points. ZDF/TRO animals had lower cholesterol than diabetic ZDF animals, but the difference was only significant at the 12-week time point (Table 1).

Troglitazone prevents mesangial expansion in diabetic animals

Examination of kidney sections from ZL animals from all age groups showed that the majority of the glomeruli present in these animals were normal with respect to BM-CSPG distribution (Fig. 1A). Morphometry of glomeruli from the ZL animals showed a trend for increasing size of the glomeruli as the animals aged (Table 2). In accord, the mesangial area in these animals also increased concomitantly at one, three, and six months. In addition, none of the ZL animals examined at any time interval had any significant interstitial fibrosis.

In contrast, at one month, the glomeruli from the untreated ZDF animals appeared to be slightly hypertrophic. Morphometry studies (Table 2) showed that the area of the glomerular tuft and mesangial area in these animals also increased over the one- to six-month time interval. Accordingly, glomerular volume followed the same trend. All of the former morphologic parameters were significantly different from the ZL animals at all time intervals examined ($P < 0.001$). By three months of untreated diabetes, glomeruli that were in the sclerotic process were markedly evident, and the presence of tubular damage was noted, becoming progressively worse by six months (Figs. 1B, 2, and 3). Interestingly, in the connective tissue between the affected tubules, BM-CSPG–associated immunofluorescence was present, a finding that had not been reported in the earlier studies in the type 1 diabetic rat model (Fig. 3).

During the same time course, morphometry of glomeruli from ZDF/TRO animals showed that glomerular hypertrophy and mesangial hypertrophy also occurred in these animals, albeit not as great of a change as seen in the ZDF animals (Table 2). When glomerular areas of all three groups were normalized to body weight, the glomeruli of the ZDF/TRO animals were not hypertrophied (data not shown). The mesangial volume fraction (V_vMes) increased in both groups of diabetic animals over the duration of the study. However, the glomeruli from the ZDF showed the greatest amount of expansion over time (41.3% increase) when compared with the ratio seen in the control animals increase for ZDF/TRO animals (3.44% increase). The glomeruli from the ZL animals showed no change in mesangial expansion during the same time period.

To assess the functional impact of troglitazone administration in these animals, urinary protein levels were measured for animals in all groups at six months. The ZL animals had the least amount of protein (7.8 mg protein/mg creatinine). The urine of the ZDF/TRO animals had a slight elevation in urinary protein (12.6 mg protein/mg creatinine; Table 2), whereas the urine of the ZDF animals had a substantial amount of protein present (28.6 mg protein/mg creatinine). These urinary protein levels, in turn, showed a high degree of correlation (0.986) with the observed mesangial expansion.

Troglitazone normalizes pancreatic islet morphology

Figure 4 shows pancreatic islet morphology from lean control animals, diabetic animals, and troglitazone-treated animals. As has been previously noted, disruption of islet morphology can be seen in the sections taken from untreated diabetic animals (Fig. 4C, D) [31]. There is a marked hyperplasia of the β-cell mass with a deposition

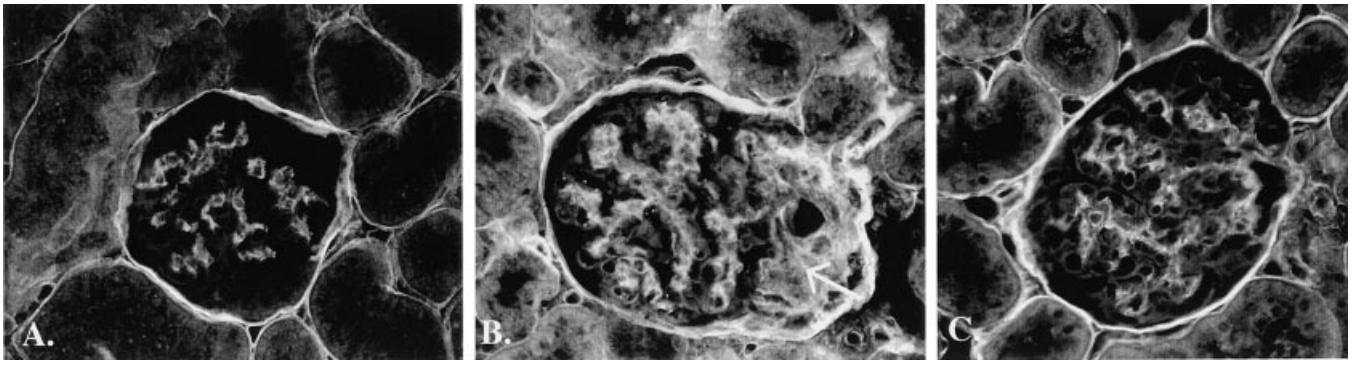

Fig. 1. Photomicrographs of sections of kidney from ZL (*A*), ZDF (*B*), and ZDF/TRO (*C*) animals (t = 6 months) immunostained with a monoclonal antibody against BM-CSPG. In both (A) and (C), the distribution of BM-CSPG in the glomeruli has an arborized appearance, reflecting the normal pattern of mesangial matrix within the glomerulus. In contrast, in a glomerulus from a ZDF animal (B), an area of focal segmental sclerosis can be seen (arrow); BM-CSPG immunostaining occurs uniformly throughout the sclerotic region. Final magnification ×400.

Table 2. Morphologic parameters

Time from onset of study	Animal group	Tuft area	Mesangial area	Glomerular volume	V_vMes	Urinary protein
		μ^2		$10^6\ \mu^3$		*mg protein/mg creatinine*
1 Month	Lean control	20912 ± 114	6356 ± 113	3.82 ± 0.032	0.29 ± 0.0045	ND
	Untreated	25734 ± 112[ad]	8467 ± 105[ad]	5.20 ± 0.034[ad]	0.33 ± 0.0037[ad]	ND
	Troglitazone treated	21877 ± 112[a]	6068 ± 95	4.08 ± 0.032[a]	0.28 ± 0.0039[a]	ND
3 Months	Lean control	23569 ± 217	6185 ± 89	4.65 ± 0.134	0.26 ± 0.0032	ND
	Untreated	31365 ± 177[ad]	11195 ± 121[ad]	7.02 ± 0.059[ad]	0.36 ± 0.0037[ad]	ND
	Troglitazone treated	27735 ± 139[a]	7110 ± 77[a]	5.84 ± 0.044[a]	0.26 ± 0.0024	ND
6 Months	Lean control	25529 ± 138	7522 ± 103	5.14 ± 0.042	0.29 ± 0.0035	8.3 ± 2
	Untreated	35631 ± 204[ad]	14620 ± 154[ad]	8.52 ± 0.073[ad]	0.42 ± 0.0038[ad]	28.6 ± 5[ac]
	Troglitazone treated	31988 ± 162[a]	9676 ± 106[a]	7.23 ± 0.055[a]	0.30 ± 0.0029	12.6 ± 3

Data are ± SEM. Abbreviations are: A_t, tuft area; A_m, mesangial area; V_vMes, mesangial volume fraction.
[a] $P < 0.001$ compared to ZL
[b] $P < 0.05$ compared to ZL
[c] $P < 0.05$ compared to troglitazone-treated obese ZDF
[d] $P < 0.001$ compared to troglitazone-treated obese ZDF

of collagen (Fig. 4C). Furthermore, the α cells and δ cells no longer form a capsule surrounding the islet (Fig. 4D). As can be seen in Figure 4E and F, troglitazone-treated animals have islet morphology that is indistinguishable from control animals with α-cells and δ-cells encapsulating a tight β-cell mass. Similar observations have been reported previously for regranulation and morphology of islets in the diabetic db/db mouse [40].

Islet architectural changes correlate with β-cell failure in the ZDF rat [31, 41]. Preservation of normal metabolic parameters in these animals is reflected in the preservation of normal islet architecture and the retention of β-cell function.

DISCUSSION

Our study shows that the insulin-sensitizing effect of troglitazone treatment has long-term positive effects on virtually every derangement accompanying diabetes in the ZDF rat. Our study shows that troglitazone treatment prevents the onset of hyperglycemia in the ZDF rat for at least six months. Troglitazone treatment ameliorates both hypertriglyceridemia and hyperinsulinemia when maintained at a 400 mg/kg dose. One possible mechanism for these effects is that troglitazone-induced improvement of peripheral insulin resistance results in marked improvement in islet morphology and normalization of β-cell function. This may be due to alleviation of demands on the β cell for increased insulin production to compensate for insulin resistance.

Because of the significant morbidity and mortality associated with the onset and progression of nephropathy in individuals afflicted with diabetes mellitus, one aspect of our study focused on the efficacy of therapeutic intervention with troglitazone in either slowing or preventing this process from occurring. Although thickening of the GBM has been viewed as an indicator of structural changes in the early stages of the disease, its onset (<3 years duration) does not always correlate with the onset of functional changes as indicated by markers such as microalbuminuria [1]. Mesangial expansion, a structural change that has been thought to occur later in the pro-

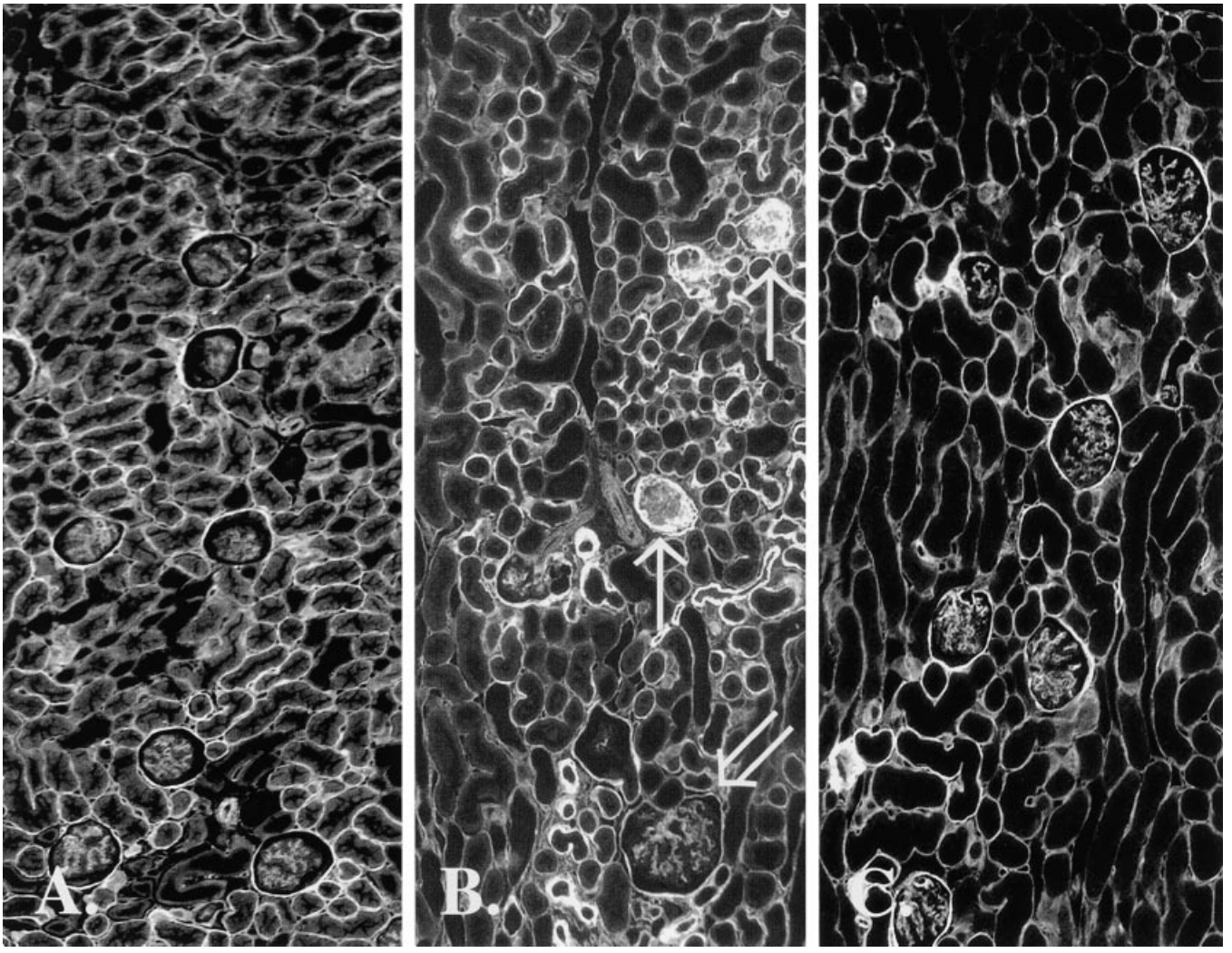

Fig. 2. A low-power micrograph showing BM-CSPG immunostaining of sections of kidney from (*A*) ZL (control animals), (*B*) ZDF (untreated diabetic animals), and (*C*) ZDF/TRO (troglitazone-treated animals). All sections in this panel are from animals from the six-month time interval. The section from the untreated diabetic animal (B) shows hypertrophic (open arrow) and sclerosed glomeruli (arrows), and tubules with distorted, thickened basement membranes are prominently stained with the BM-CSPG antibodies. Such damage was not evident in any of the sections examined from either ZL (A) or ZDF/TRO (B). Final magnification ×400.

gression of diabetic nephropathy, correlates with both microalbuminuria and macroalbuminuria [1].

Mesangial expansion, the increase in the volume of the mesangium relative to the volume of the glomerular capillary tuft, can also be viewed in another light, that is, the gradual abrogation of the discrete organization of extracellular matrix domains in the glomerulus. Early ultrastructure studies first demonstrated that the two closely approximated extracellular matrices, that is, the mesangial matrix and GBM, were morphologically distinct in appearance [42, 43]. Subsequent immunohistochemical studies now show that these domains are unique in their construction, in that each matrix domain is composed in part of specific matrix molecules or their isoforms [34, 44–46].

In the context of the current study, the extent of glomerular immunostaining of BM-CSPG was used to demarcate the extent of mesangial matrix present within the glomeruli of normoglycemic and diabetic animals. The rationale for using BM-CSPG as a marker was twofold. First, our previous immunohistochemistry studies in the type 1 diabetic rat demonstrated that discrete changes in the pattern of immunolocalization of BM-CSPG occurred very early in the onset of the disease (t = 1 month duration of uncontrolled diabetes mellitus) [30]. Second, the abnormal association of BM-CSPG with the glomerular capillary wall was, in turn, associated with focal displacement/detachment of endothelial cells from the GBM as well as effacement of podocyte foot processes. Thus, we believe that the marker was sensitive

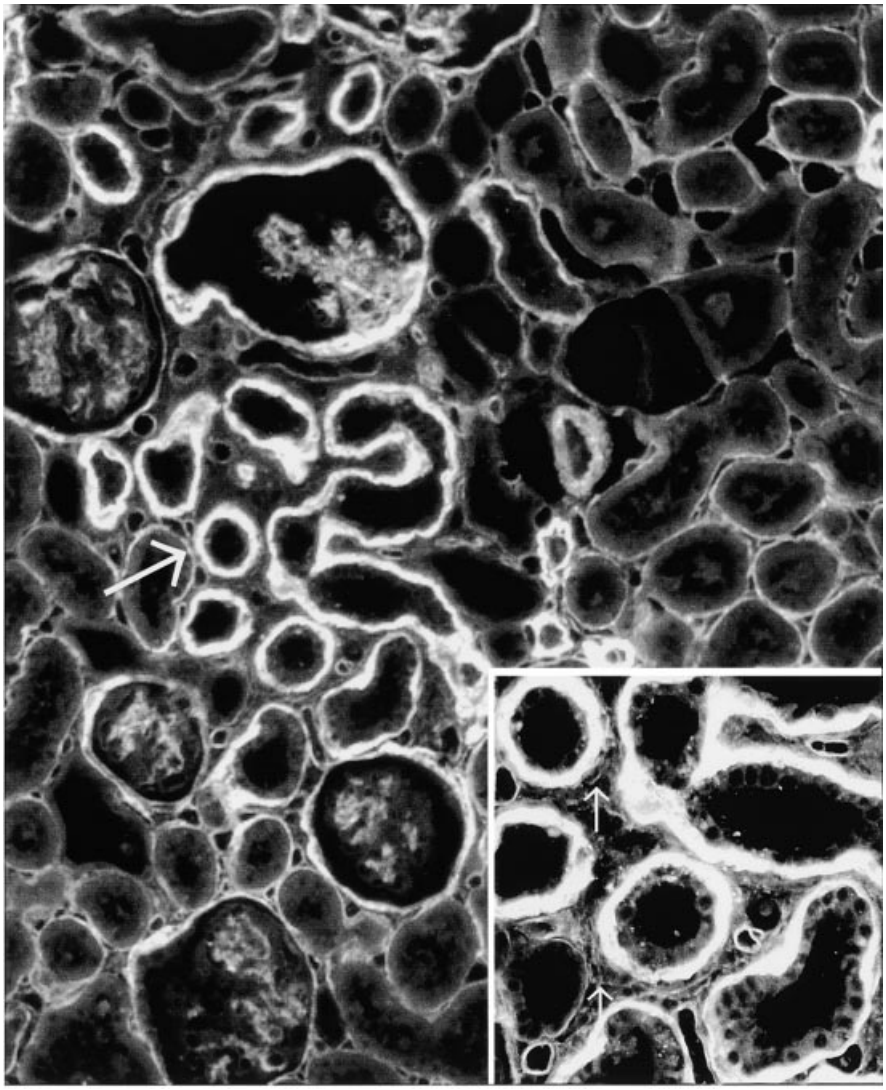

Fig. 3. A higher magnification of an area of tubular damage from the kidney of an ZDF animal with six month duration of uncontrolled diabetes mellitus. Several glomeruli are present in this field surrounding an area of damaged tubules (arrow). The basement membranes in this cluster of tubules are abnormally thickened when compared with the basement membranes of tubules directly adjacent (right) to the damaged cluster. Interestingly, besides staining the capillaries that lie in the interstitial areas between the damaged tubules (inset, higher magnification), BM-CSPG immunostains irregular strands of material that do not appear to associated with either the tubular or capillary basement membranes (arrows). Final magnification ×100; inset ×400.

enough to detect subtle alterations in glomerular infrastructure at a time far before any functional deficits could be measured.

Consistent with the results of other studies, our measurements show that the response to uncontrolled diabetes mellitus in this animal model results in glomerular hypertrophy and mesangial expansion. However, our study shows that this response may not be sequential as previously thought: that is, that glomerular hypertrophy occurs first, and is followed later by mesangial expansion. Rather, our data indicate that glomerular hypertrophy and mesangial expansion occur in the diabetic rats almost concurrently, as early as one month's duration of untreated diabetes mellitus. The discordance of our data with earlier studies can be readily explained by the fact that those prior studies relied solely on morphology/stereology to detect mesangial expansion and did not have mesangium-specific probes to assist in demarcating the true extent of mesangial matrix [23, 25–27]. Because of this recent technology, we were able to measure readily the extent of mesangium in a significant number of glomeruli ($N = 200$ per animal). Our data show that at one month, mesangial expansion in the untreated diabetic animals, as indicated by V_vMes, was significantly different ($P < 0.001$) than either the normoglycemic or troglitazone-treated diabetic animals. To our knowledge, this is the first report of the measurement of a consistent, significant mesangial expansion occurring in the earliest stages of nephropathy. Our data suggest that mesangial expansion should not be categorized as a late-stage change, since its genesis may actually occur in the earliest stages of the progression of glomerulosclerosis.

In the case of the ZDF/TRO animals, glomerular hypertrophy occurred at all time intervals when compared with control animals ($P < 0.001$); however, the degree of glomerular hypertrophy for the ZDF/TRO animals

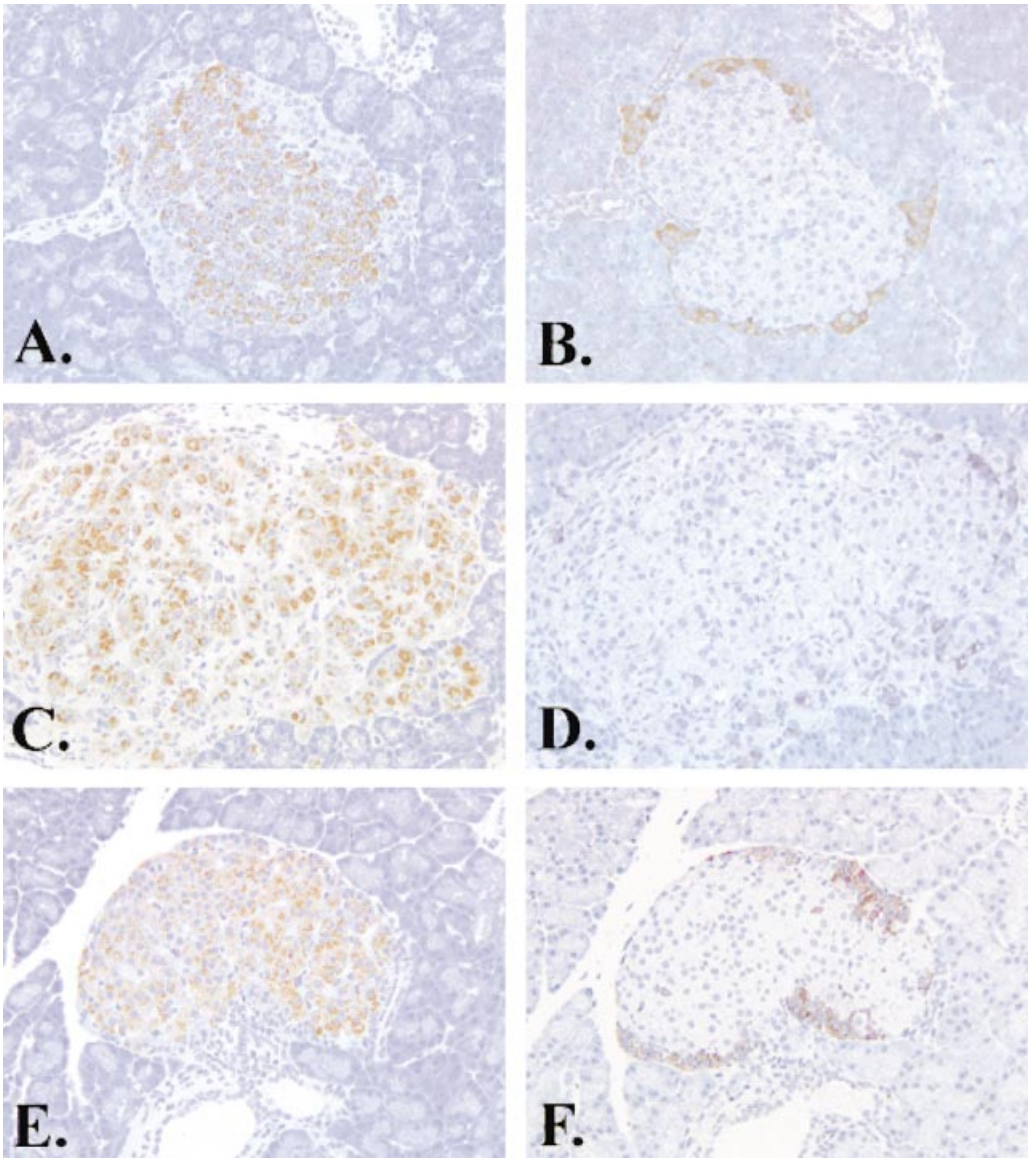

Fig. 4. Islet morphology of ZL rats (*A* and *B*), ZDF rats (*C* and *D*), and ZDF/TRO rats (*E* and *F*) at 18 weeks of age. Adjacent sections of pancreas were stained with antibodies to insulin (A, C, and E) for β cells or glucagon/somatostatin (B, D, and F) for α and δ cells. The pancreata from the ZDF animals (C and D) are hypertrophied in comparison to those from the ZL (A and B) and ZDF/TRO (E and F). Final magnification ×400.

was less that that seen in the ZDF animals ($P < 0.001$) at all time intervals. Given the fact that the thiazolidinediones might have a direct effect on vascular smooth muscle tone [47, 48], it may be possible that the glomerular hypertrophy seen in the ZDF/TRO animals may be due to changes in vascular tone at the level of the afferent arteriole that that may be entirely independent of the diabetic pathophysiology. However, this observation remains to be investigated in future studies. Although glomerular hypertrophy has been associated as a factor of disease progression, the mesangial expansion data indicate that progression beyond the initial hypertrophy did not occur in the ZDF/TRO animals. The lack of progression in the ZDF/TRO animals was also reflected in the fact that a significant difference in proteinuria was not seen between the ZL and ZDF/TRO animals ($P = 0.3932$) at the six-month interval.

One question raised by our study and that of Buckingham et al is, "how do the thiazolidinediones exert their renoprotective effects?" [49]. The thiazolidinediones have direct effects on two significant metabolic abnormalities that occur in diabetes mellitus that, when properly controlled, are known to ameliorate diabetic complications. First, the thiazolidinediones are capable of normalizing hyperglycemia in the type 2 diabetic patient. A substantial body of literature demonstrates that pro-

longed hyperglycemic conditions directly affect the biosynthesis/degradation [50, 51] and glycation status [52] of extracellular matrices secreted by mesangial cells. Second, thiazolidinediones are capable of controlling the abnormal lipid metabolism in type 2 diabetic animals. Other investigations in the obese Zucker rat, a hyperinsulinemic but normoglycemic animal model of obesity, indicate that control of defects in lipid metabolism plays an important role in preventing the progression of glomerulosclerosis in that model [49, 53–57]. Thus, it is logical to assume that the prevention of diabetic complications by the thiazolidinediones is related to the systemic control of those very factors that accelerate progression of complications.

However, there is other evidence that the thiazolidinediones may also have a direct effect on glomerular physiology. A preliminary report from Isshiki et al (abstract; *J Am Soc Nephrol* 9:S844, 1998) showed troglitazone suppressed an increase in transforming growth factor-β, fibronectin, and αI (IV) collagen expression in glomeruli from diabetic animals, possibly by preventing the activation of protein kinase C and mitogen-activated protein kinase. Both pathways have been shown to affect the expression of extracellular matrix proteins. Preliminary data from reverse transcription-polymerase chain reaction studies from our laboratory (K.J. McCarthy and R. Routh, unpublished data) of isolated rat mesangial cells grown in culture indicate that mesangial cells express both peroxisome proliferator-activated receptor (PPAR) γ and α in culture. This raises the possibility that the thiazolidinediones could also exert their effects locally within the glomerulus by directly affecting gene expression in mesangial cells via activation of the PPAR γ and or modulating PPAR α signaling pathways.

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Reprint requests to Kevin J. McCarthy, Ph.D., Department of Pathology, Louisiana State University School of Medicine, 1501 King's Highway, Shreveport, Louisiana 71130-3932, USA.
E-mail: kmccar2@lsuhsc.edu

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